10-23-00

A

Practitioner's Docket No. 700953-45394C **PATENT**

Preliminary Classification:
Proposed Class:
Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P. Section 601, 7th ed.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of
Inventor(s): Jeffrey, SCHLOM; Dennis, PANICALI

CERTIFICATION UNDER 37 C.F.R. SECTIONS 1.8(a) AND 1.10*
*(When using Express Mail, the Express Mail label number is **mandatory**; Express Mail certification is optional.)*

I hereby certify that, on the date shown below, this correspondence is being:

MAILING

[ ] deposited with the United States Postal Service in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

37 C.F.R. Section 1.8(a) **37 C.F.R. Section 1.10***

[ ] with sufficient postage as first class mail.

[x] as "Express Mail Post Office to Address" Mailing Label No. ________ **(mandatory)** EL565095244US

TRANSMISSION

[ ] transmitted by facsimile to the Patent and Trademark Office (703) ________.

Date: October 20, 2000

Patricia Turner
Signature

Patricia Turner
(type or print name of person certifying)

***WARNING:** *Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. Section 1.10(b).*
*"Since the filing of correspondence under [Section] 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

For (title):GENERATION OF IMMUNE RESPONSES TO PROSTATE-SPECIFIC ANTIGEN (PSA)

1. Type of Application

This new application is for a(n)

[ ] Original (nonprovisional)
[ ] Design
[ ] Plant
[ ] Divisional.
[X] Continuation.
[ ] Continuation-in-part (C-I-P).

2. Benefit of Prior U.S. Application(s) (35 U.S.C. Sections 119(e), 120, or 121)

[X] The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed

A. Required for Filing Date under 37 C.F.R. Section 1.53(b) (Regular) or 37 C.F.R. Section 1.153 (Design) Application

44_____Pages of Specification
3______Pages of Claims
2______Sheets of Drawing

B. Other Papers Enclosed

1______Pages of declaration and power of attorney
1______Pages of Abstract
_______Other

4. Additional Papers Enclosed

[ ] Amendment to claims

[ ] Cancel in this applications claims __________________before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

[ ] Add the claims shown on the attached amendment. (Claims added have been

numbered consecutively following the highest numbered original claims.)

[ ] Preliminary Amendment
[ ] Information Disclosure Statement (37 C.F.R. Section 1.98)
[ ] Form PTO-1449 (PTO/SB/08A and 08B)
[ ] Citations
[ ] Declaration of Biological Deposit
[ ] Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.
[ ] Authorization of Attorney(s) to Accept and Follow Instructions from Representative
[ ] Special Comments
[ ] Other

5. Declaration or Oath (including power of attorney)

[X] Enclosed

Executed by

[X] inventor(s).
[ ] legal representative of inventor(s). 37 C.F.R. Section 1.42 or 1.43.
[ ] joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

[ ] This is the petition required by 37 C.F.R. Section 1.47 and the statement required by 37 C.F.R. Section 1.47 is also attached. See item 13 below for fee.

[ ] Not Enclosed.

[ ] Application is made by a person authorized under 37 C.F.R. 1.41 on behalf of *all* the above named inventor(s).

[ ] Showing that the filing is authorized.
(not required unless called into question. 37 C.F.R. Section 1.41(d))

6. Inventorship Statement

The inventorship for all the claims in this application are:

[ ] The same.

or

[ ] Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,
[ ] is submitted.
[ ] will be submitted.

7. **Language**

[X] English
[ ] Non-English

[ ] The attached translation includes a statement that the translation is accurate. 37 C.F.R. Section 1.52(d).

8. **Assignment**

[ ] An assignment of the invention to ______________________________

____

[ ] is attached. A separate [ ] "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or [ ] FORM PTO 1595 is also attached.
[ ] will follow.

9. **Certified Copy**

Certified copy(ies) of application(s)

Country	Appln. no.	Filed
Country	Appln. no.	Filed
Country	Appln. no.	Filed

from which priority is claimed
[ ] is (are) attached.
[ ] will follow.

10. **Fee Calculation** (37 C.F.R. Section 1.16)

A. [X] Regular application

CLAIMS AS FILED

Claims	Number Filed	Basic Fee Allowance	Number Extra	Rate	Basic Fee 37 C.F.R. Section 1.16(a) $710.00
Total Claims (37 C.F.R. Section 1.16(c))		18 - 20 =	x	$18.00	
Independent Claims (37 C.F.R. Section 1.16(b))		4 - 3 =	x	$78.00	$78.00
Multiple Dependent Claim(s), if any (37 C.F.R. Section 1.16(d))		+	$260.00		

[ ] Amendment cancelling extra claims is enclosed.
[ ] Amendment deleting multiple-dependencies is enclosed.
[ ] Fee for extra claims is not being paid at this time.

Filing Fee Calculation $ 788.00

B. [ ] Design application
($310.00--37 C.F.R. Section 1.16(f))
Filing Fee Calculation $__________

C. [ ] Plant application
($480.00--37 C.F.R. Section 1.16(g))
Filing Fee Calculation $__________

11. Small Entity Statement(s)

[ ] Statement(s) that this is a filing by a small entity under 37 C.F.R. Section 1.9 and 1.27 is (are) attached.

[ ] Status as a small entity was claimed in prior application ______/____________, filed on __________________ from which benefit is being claimed for this application under:

35 U.S.C. Section [ ] 119(e),
[ ] 120,
[ ] 121,
[ ] 365(c),

and which status as a small entity is still proper and desired.

[ ] A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of **A**, **B** or **C** above) $ 788.00

12. Request for International-Type Search (37 C.F.R. Section 1.104(d))

[ ] Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. Fee Payment Being Made at This Time

[ ] Not Enclosed

[ ] No filing fee is to be paid at this time.
(This and the surcharge required by 37 C.F.R. Section 1.16(e) can be paid subsequently.)

[X] Enclosed

[X] Filing fee $ 788.00

[ ] Recording assignment
($40.00; 37 C.F.R. Section 1.21(h))
(See attached "COVER SHEET FOR ASSIGNMENT ACCOMPANYING NEW APPLICATION.") $

[ ] Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached
($130.00; 37 C.F.R. Sections 1.47 and 1.17(i))$

[ ] For processing an application with a specification in a non-English language
($130.00; 37 C.F.R. Sections 1.52(d) and 1.17(k))$

[ ] Processing and retention fee
($130.00; 37 C.F.R. Sections 1.53(d) and 1.21(l))$

[ ] Fee for international-type search report
($40.00; 37 C.F.R. Section 1.21(e)) $

Total Fees Enclosed $ 788.00

14. Method of Payment of Fees

[X] Check in the amount of $ 788.00 .

[ ] Charge Account No. __________ in the amount of $__________.
A duplicate of this transmittal is attached.

15. Authorization to Charge Additional Fees

[X] The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. 50-0850 .

- [X] 37 C.F.R. Section 1.16(a), (f) or (g) (filing fees)
- [ ] 37 C.F.R. Section 1.16(b), (c) and (d) (presentation of extra claims)
- [ ] 37 C.F.R. Section 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)
- [ ] 37 C.F.R. Section 1.17(a)(1)-(5) (extension fees pursuant to Section 1.136(a).
- [ ] 37 C.F.R. Section 1.17 (application processing fees)
- [ ] 37 C.F.R. Section 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. Section 1.311(b))

16. Instructions as to Overpayment

[X] Credit Account No. 50-0850.

[ ] Refund

SIGNATURE OF PRACTITIONER

Reg. No. 34,235

David S. Resnick
(type or print name of practitioner)

Tel. No.: (617) 345-6057

101 Federal Street
P.O. Address

Customer No.:

Boston, MA 02110

[X] **Incorporation by reference of added pages**

(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)

[X] Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed
Number of pages added 1

[ ] Plus Added Pages for Papers Referred to in Item 4 Above
Number of pages added ______

[ ] Plus added pages deleting names of inventor(s) named on prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.
Number of pages added ______

[ ] Plus "Assignment Cover Letter Accompanying New Application"
Number of pages added ______

[ ] **Statement Where No Further Pages Added**

(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)

[ ] This transmittal ends with this page.

JC813 U.S. PTO
09/693121
10/20/00

ADDED PAGE(S) FOR SPECIAL COMMENTS FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S)N IS CLAIMED

17. Relate back

A. 35 U.S.C. 119(e)

B. 35 U.S.C. 120, 121 and 365(c)

[X] "This application is a

[X] continuation
[ ] continuation-in-part
[ ] divisional

of copending application(s)

[X] application number 08/500,306 filed on July 10, 1995.

[ ] International Application ____________ filed on ____________ and which designated U.S."

[ ] "The non-provisional application designed above, namely application number 08/500,306 filed on July 10, 1995, claims benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	**FILING DATE**
______/______________	________________"
______/______________	________________"
______/______________	________________"

[ ] Where more than one reference is made above please combine all references into one sentence.

Added page 1

Practitioner's Docket No. 953-45394-CPA-2C *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: SCHLOM, et al.

Application No.: Group No.:
Filed: Submitted herewith Examiner:

For: GENERATION OF IMMUNE RESPONSES TO PROSTATE-SPECIFIC ANTIGEN (PSA)

Assistant Commissioner for Patents
Washington, D.C. 20231

EXPRESS MAIL CERTIFICATE

"Express Mail" label number EL565095244US
Date of Deposit 10/20/2000

I hereby state that the following *attached* papers or fees

New Application Transmittal
Copy of the Application No. 08/500,306 including 47 pages of Specification, Abstract, 2 pages of Sequence Listing, 2 sheets of Drawings;
Copy of Declaration and Power of Attorney;
Check in the sum of $788.00; and
Return Receipt Postcard and Change of Attorney's Address in Application.

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. section 1.10, on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Patricia Turner

Patricia Turner

Signature of person mailing paper or fee

GENERATION OF IMMUNE RESPONSES TO PROSTATE-SPECIFIC ANTIGEN (PSA)

FIELD OF THE INVENTION

The present invention relates generally to generation of cellular and humoral immune responses to a mammalian prostate-specific antigen (PSA).

BACKGROUND OF THE INVENTION

Cancer of the prostate is the most commonly diagnosed cancer in men and is the second most common cause of cancer death (Carter, et al., 1990; Armbruster, et al., 1993). If detected at an early stage, prostate cancer is potentially curable. However, a majority of cases are diagnosed at later stages when metastasis of the primary tumor has already occurred (Wang, et al., 1982). Even early diagnosis is problematic because not all individuals who test positive in these screens develop cancer. Present treatment for prostate cancer includes radical prostatectomy, radiation therapy, or hormonal therapy. No systemic therapy has clearly improved survival in cases of hormone refractory disease. With surgical intervention, complete eradication of the tumor is not always achieved and the observed re-occurrence of the cancer (12-68%) is dependent upon the initial clinical tumor stage (Zietman, et al., 1993). Thus, alternative methods of treatment including prophylaxis or prevention are desirable.

Prostate specific antigen (PSA) is a 240 amino acid member of the glandular kallikrein gene family. (Wang, et al., 1982; Wang, et al., 1979; Bilhartz, et al., 1991). PSA is a serine protease, produced by normal prostatic tissue, and secreted exclusively by the epithelial cells lining prostatic acini and ducts (Wang, et al., 1982; Wang, et al., 1979; Lilja, et al., 1993). Prostate specific antigen can be detected at low levels in the sera of healthy males without clinical evidence of prostate cancer.

However, during neoplastic states, circulating levels of this antigen increase dramatically, correlating with the clinical stage of the disease (Schellhammer, et al., 1993; Huang, et al., 1993; Kleer, et al., 1993; Oesterling, et al., 1991). Prostate specific antigen is now the most widely used marker for prostate cancer. The tissue specificity of this antigen makes PSA a potential target antigen for active specific immunotherapy (Armbruster, et al., 1993; Brawer, et al., 1989), especially in patients who have undergone a radical prostatectomy in which the only PSA expressing tissue in the body should be in metastatic deposits. Recent studies using in-vitro immunization have shown the generation of CD4 and CD8 cells specific for PSA (Peace et al., 1994; Correale et al., 1995). However, although weak natural killer cell responses have been occasionally documented in prostate cancer patients (Choe, et al., 1987), attempts to generate an *in vivo* immune response have met with limited success. For example, several attempts to actively immunize patients with prostate adenocarcinoma cells admixed with Bacillus Calmette-Guerin (BCG) have shown little or no therapeutic benefit (Donovan, et al., 1990). The ability to elicit an immune response as a result of exposure to PSA in vivo would be extremely useful.

Vaccinia virus has been used in the world-wide eradication of smallpox. This virus has been shown to express a wide range of inserted genes, including several tumor associated genes such as p97, HER-2/neu, p53 and ETA (Paoletti, et al., 1993). Other pox viruses that have been suggested as useful for expression of multiple genes include avipox such as fowl pox. Cytokines expressed by recombinant vaccinia virus include IL-1, IL-2, IL-5, IL-6, TNF-α and IFN-γ (Paoletti, et al., 1993). Recombinant pox viruses, for example vaccinia viruses, are being considered for use in therapy of cancer because it has been shown in animal models that the co-presentation of a weak immunogen with the highly immunogenic poxvirus

proteins can elicit a strong immune response against the inserted gene product (Kaufman, et al., 1991; Paoletti, et al., 1993; Kantor, et al., 1992a; Kantor, et al., 1992b; Irvine, et al., 1993; Moss, et al., 1993). A recombinant vaccinia virus containing the human carcinoembryonic antigen gene has just completed phase 1 clinical trials in carcinoma patients with no evidence of toxicity other than that observed with the wild type smallpox vaccine (Kantor, et al., 1992b).

Currently, models for the evaluation of prostate therapeutics include the canine (McEntee, et al., 1987), and the Dunning rat (Isaacs, et al., 1986); neither of these models, however, are practical for the study of PSA-recombinant vaccines due to the very low homology of rat and canine PSA to human PSA (Karr, et al., 1995; Schroder, et al., 1982). In contrast, the prostate gland of the rhesus monkey is structurally and functionally similar to the human prostate (Wakui, et al., 1992). At the molecular level, there is 94% homology between either the amino acid or nucleic acid sequences of rhesus PSA (Gauthier, et al., 1993) and those sequences of human prostate specific antigen (Karr, et al., 1995; Lundwall, et al., 1987). Thus, human PSA is essentially an autoantigen in the rhesus monkey. Accordingly, the rhesus monkey can serve as a model for autologous anti-PSA immune reactions.

SUMMARY OF THE INVENTION

We have discovered that by using a recombinant viral vector, preferably a pox virus vector having at least one insertion site containing a DNA segment encoding prostate-specific antigen (PSA), or a cytotoxic T-cell eliciting epitope thereof, operably linked to a promoter capable of expression in the host, a specific humoral and cellular immune response to PSA can be generated. The method preferably comprises introducing a sufficient amount of the recombinant pox virus vector into a host to

stimulate the immune response, and contacting the host with additional PSA at periodic intervals thereafter. The additional PSA, or a cytotoxic T-cell eliciting epitope thereof, may be added by using a second pox virus vector from a different pox genus. In another embodiment, additional PSA can be added by contacting the host with PSA by a variety of other methods, including in one preferred embodiment adding PSA. The PSA may be formulated with an adjuvant or in a liposomal formulation.

In a further embodiment, an immune response to PSA can be generated by contacting the host initially with a sufficient amount of PSA, or a cytotoxic T-cell eliciting epitope thereof, to stimulate an immune response and at periodic intervals thereafter contacting the host with additional PSA. The additional PSA, or a cytotoxic T-cell generating fragment thereof, may be added using a pox virus vector as discussed above.

We have also discovered that human cytotoxic T-cells specific for PSA can be produced using a cytotoxic T-cell eliciting epitope of the PSA and that these cells have the ability to lyse PSA-expressing human prostate carcinoma cells.

As used herein the term "prostate specific antigen" includes the native protein whether purified from a native source or made by recombinant technology, as well as any polypeptide, mutein or portion derived therefrom that is capable of generating an immune response to a native conformationally correct PSA. For example, one can make conservative amino acid substitutions in the molecule without adversely affecting the ability to use the recombinant to generate an antibody that will also recognize native PSA.

The pox virus is preferably selected from the group of pox viruses consisting of suipox, avipox, capripox and orthopox virus. Preferred orthopox include vaccinia, rabbit pox and raccoon pox. Preferred avipox includes fowlpox, canary pox and pigeon pox. A more preferred avipox is fowlpox. The preferred suipox is swinepox.

Vaccinia viral vectors may elicit a strong antibody response. Thus while numerous boosts with vaccinia vectors are possible, its repeated use may not be preferred in certain instances. We have discovered that by using pox from different genera to boost, this sensitivity problem can be minimized. In accordance with the present invention, in order to avoid such problems, preferably, when the first or initial pox virus vector is vaccinia, the second and subsequent pox virus vectors are selected from the pox viruses from a different genus such as suipox, avipox, capripox or an orthopox immunogenically distinct from vaccinia.

Adjuvants include, for example, RIBI Detox, QS21, and incomplete Freund's adjuvant. Liposomal formulations can also be used.

Human cytotoxic T-cells specific for PSA produced in accordance with the present invention can be isolated from a human host. These cells can be used in drug assays, used to map cytotoxic T-cells eliciting antigen epitopes or in adoptive cell therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a Western blot of PSA from rV-PSA infected BSC-40 cells. Lanes 2-4 are extracts from supernatant fluid from cells infected overnight with rV-PSA at an MOI of 1, while Lanes 7-9 are extracts from the corresponding infected cells. Lanes 1 and 7 are supernatant extracts and cell extracts from V-Wyeth infected cells. Blot was developed using a

specific MAb for human PSA. This blot illustrates that cells infected with rV-PSA authentically express and secrete the 33 kD PSA protein.

Figures 2A, 2B and 2C show the manifestation of rV-PSA immunization. In Figure 2A, the area of lesions was measured 7 days following each inoculation of rhesus monkeys with either V-Wyeth (open circles) or rV-PSA (closed circles). In Figure 2B, the duration of the lesion was monitored as time of scab disappearance. In Figure 2C, the extent of lymph node swelling was recorded and characterized as very swollen (3+), i.e., more than two axillary nodes swollen; swollen (2+), i.e., one or two nodes easily palpable; marginally swollen (1+), i.e., one node was barely palpable; or not swollen (0), 7 days following inoculation with vaccinia virus. Each symbol represents one monkey.

DETAILED DESCRIPTION OF THE INVENTION

We have induced an immune response specific to PSA in the rhesus monkey model by placing the PSA gene into a recombinant viral vector, i.e, a pox vector such as vaccinia virus.

Additionally, an immune response to PSA can be generated by contacting the host initially with a sufficient amount of PSA, or a cytotoxic T-cell eliciting epitope thereof, to stimulate an immune response and at periodic intervals thereafter contacting the host with additional PSA. The additional PSA, or a cytotoxic T-cell generating fragment thereof, may be added using a pox virus vector.

A DNA fragment encoding the open reading frame of human PSA can be obtained, for example, from total RNA extracted from the human metastatic prostate adenocarcinoma cell line, LNCaP.FGC (CRL 1740, American Type Cell Culture (ATCC), Rockville, MD) by reverse

transcriptase PCR using PSA specific oligonucleotide primers 5' TCTAGAAGCCCCAAGCTTACCACCTGCA 3' (SEQ. ID. NO.:1), 5' TCTAGATCAGGGGTTGGCCACGATGGTGTCCTTGATCCACT 3' (SEQ. ID. NO.:2). The nucleotide sequence of the PSA cDNA has been published (Lundwall, et al., 1987).

Recombinant human PSA can be obtaining using a baculovirus expression system in accordance with the method of Bei et al., *J. Clin. Lab. Anal.*, 9:261-268 (1995), the disclosure of which is herein incorporated by reference.

Viral vector

Basic techniques for preparing recombinant DNA viruses containing a heterologous DNA sequence encoding the carcinoma self-associated antigen or cytotoxic T-cell eliciting epitope are known to the skilled artisan and involve, for example, homologous recombination between the viral DNA sequences flanking the DNA sequence in a donor plasmid and homologous sequences present in the parental virus (Mackett, et al., *Proc. Natl. Acad. Sci. USA* 79:7415-7419 (1982)). For example, recombinant viral vectors such as a pox viral vector can be used in delivering the gene. The vector can be constructed for example by steps known in the art, e.g. analogous to the methods for creating synthetic recombinants of the fowlpox virus described in U.S. Patent No. 5,093,258, the disclosure of which is incorporated herein by reference. Other techniques include using a unique restriction endonuclease site that is naturally present or artificially inserted in the parental viral vector to insert the heterologous DNA.

Pox viruses useful in practicing the present invention include orthopox, suipox, avipox and capripox virus.

Orthopox include vaccinia, ectromelia and raccoon pox. The preferred orthopox is vaccinia.

Avipox includes fowlpox, canary pox and pigeon pox. The preferred avipox is fowlpox.

Capripox include goatpox and sheeppox.

A preferred suipox is swinepox.

Other viral vectors that can be used include other DNA viruses such as herpes virus and adenoviruses, and RNA viruses such as retroviruses and polio.

For example, the DNA gene sequence to be inserted into the virus can be placed into a donor plasmid, e.g., an *E. coli* plasmid construct, into which DNA homologous to a section of DNA such as that of the insertion site of the poxvirus where the DNA is to be inserted has been inserted. Separately the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA which is the desired insertion region. With a parental pox viral vector, a pox promoter is used. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria and isolated. Preferably, the plasmid also contains an origin of replication such as the *E. coli* origin of replication, and a marker such as an antibiotic resistance gene for selection and propagation in *E. coli.*

Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, e.g., chick embryo fibroblasts,

along with the parental virus, e.g., poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively results in a recombinant poxvirus modified by the presence of the promoter-gene construct in its genome, at a site which does not affect virus viability.

As noted above, the gene is inserted into a region (insertion region), in the virus which does not affect virus viability of the resultant recombinant virus. The skilled artisan can readily identify such regions in a virus by, for example, randomly testing segments of virus DNA for regions that allow recombinant formation without seriously affecting virus viability of the recombinant. One region that can readily be used and is present in many viruses is the thymidine kinase (TK) gene. For example, the TK gene has been found in all pox virus genomes examined [leporipoxvirus: Upton, et al., *J. Virology*, 60:920 (1986) (shope fibroma virus); capripoxvirus: Gershon, et al., *J. Gen. Virol.*, 70:525 (1989) (Kenya sheep-1); orthopoxvirus: Weir, et al., *J. Virol.*, 46:530 (1983) (vaccinia); Esposito, et al., *Virology*, 135:561 (1984) (monkeypox and variola virus); Hruby, et al., *PNAS*, 80:3411 (1983) (vaccinia); Kilpatrick, et al., *Virology*, 143:399 (1985) (Yaba monkey tumor virus); avipoxvirus: Binns, et al., *J. Gen. Virol.* 69:1275 (1988) (fowlpox); Boyle, et al., *Virology*, 156:355 (1987) (fowlpox); Schnitzlein, et al., *J. Virological Methods*, 20:341 (1988) (fowlpox, quailpox); entomopox (Lytvyn, et al., *J. Gen. Virol.* 73:3235-3240 (1992)].

In vaccinia, in addition to the TK region, other insertion regions include, for example, the HindIII M fragment.

In fowlpox, in addition to the TK region, other insertion regions include, for example, the BamHI J fragment [Jenkins, et al., *AIDS Research*

and Human Retroviruses 7:991-998 (1991)] the *Eco*RI-*Hind*III fragment, *Eco*RV-*Hind*III fragment, *Bam*HI fragment and the *Hind*III fragment set forth in EPO Application No. 0 308 220 A1. [Calvert, et al., *J. of Virol.* 67:3069-3076 (1993); Taylor, et al., *Vaccine* 6:497-503 (1988); Spehner, et al., (1990) and Boursnell, et al., *J. of Gen. Virol.* 71:621-628 (1990)].

In swinepox preferred insertion sites include the thymidine kinase gene region.

In addition to the requirement that the gene be inserted into an insertion region, successful expression of the inserted gene by the modified poxvirus requires the presence of a promoter operably linked to the desired gene, i.e., in the proper relationship to the inserted gene. The promoter must be placed so that it is located upstream from the gene to be expressed. Promoters are well known in the art and can readily be selected depending on the host and the cell type you wish to target. For example in poxviruses, pox viral promoters should be used, such as the vaccinia 7.5K, 40K or fowlpox promoters such as FPV C1A. Enhancer elements can also be used in combination to increase the level of exp-ession. Furthermore, the use of inducible promoters, which are also well known in the art, in some embodiments are preferred.

A specific immune response for PSA can be generated by administering between about 10^5-10^9 pfu of the recombinant pox virus, constructed as discussed above to a host, more preferably one uses 10^7 pfu. The preferred host is a human. At least one interval thereafter, which is preferably one to three months later, the immune response is boosted by administering additional antigen to the host. More preferably there is at least a second "boost" preferably one to three months after the first boost. The antigen may be administered using the same pox virus vector. The

antigen may preferably be administered using a second pox virus vector from a different pox genera, or may be administered directly using, for example, an adjuvant or liposome. Cytokines, e.g., IL-2, IL-6, IL-12 or co-stimulatory molecules, e.g., B7.1, B7.2, may be used as biologic adjuvants and can be administered systemically to the host or co-administered via insertion of the genes encoding the molecules into the recombinant pox vector.

Adjuvants include, for example, RIBI Detox (Ribi Immunochemical), QS21 and incomplete Freund's adjuvant.

Generation of Cytotoxic T-Cells

Cytotoxic T-cells specific for PSA can be established from peripheral blood mononuclear cells (PBMC) obtained from a host immunized as discussed above. For example, PBMC can be separated by using Lymphocyte Separation Medium gradient (Organon Teknika, Durham, NC, USA) as previously described [Boyum, et al., *Scand J. Clin Lab Invest* 21: 77-80 (1968)]. Washed PBMC are resuspended in a complete medium, for example, RPMI 1640 (GIBCO) supplemented with 10% pool human AB serum (Pel-Freeze Clinical System, Brown Dear, WI, USA), 2mM glutamine, 100 U/ml penicillin and 100 μg/ml of streptomycin (GIBCO). PBMC at a concentration of about 2×10^5 cells in complete medium in a volume of, for example, 100 μl are added into each well of a 96-well flat-bottom assay plate (Costar, Cambridge, MA, USA). The antigen or peptides are added into the cultures in a final concentration of about 50 μg/ml and incubated at 37°c in a humidified atmosphere containing 5% CO_2 for 5 days. After removal of peptide containing media, the cultures are provided with fresh human IL-2 (10U/ml) after 5 days and replenished with IL-2 containing medium every 3 days. Primary cultures are restimulated with the same peptide (50 μg/ml) on day 16. 5×10^5 irradiated (4,000 rad)

autologous PBMC are added in a volume of about 50 μl complete medium as antigen-presenting cells (APC). About five days later, the cultures are provided with human IL-2 containing medium as described previously. Cells are restimulated for 5 days at intervals of 16 days.

Epitope mapping

The cytotoxic T-cells of the present invention can be used to determine the epitope of the PSA that elicits a cytoxic T-cell. For example, one can cut the PSA into numerous peptide fragments. Alternatively, the fragments can be chemically synthesized. Cytotoxic T-cells can then be plated and different fragments added to different wells. Only T-cells which recognize one of the pre-selected peptide fragments as an epitope will continue to expand, thereby permitting ready identification.

These fragments can then be used to elicit cytotoxic T-cell instead of using the whole protein. Additionally, one can prepare other fragments containing the epitope to enhance its ability to elicit a cytoxic T-cell response. Modifications to these fragments are well known in the art and include the use of conjugates, specific amino acid residues such as cystines, etc.

Drug Assay

The cytotoxic T-cell can also be used to screen for compounds which enhance the ability of the antigen to create a cytotoxic T-cell response. For example, cytotoxic T-cells can be incubated with a selected epitope, for example, in a microtiter plate. The compound to be tested, e.g. a drug, is then added to the well and the growth of the T-cells is measured. T-cell expansion indicates that the test compound enhances the T-cell response. Such compounds can be further evaluated.

Therapy

The cytotoxic T-cell can be cultured to amplify its number and then injected back into the host by a variety of means. Generally, between 1 x 10^5 and 2 x 10^{11} cytotoxic T-cells per infusion are administered in, for example, one to three infusions of 200 to 250 ml each over a period of 30 to 60 minutes. After the completion of the infusions, the patient may be treated with recombinant interleukin-2 with a dose of 720,000 IU per kilogram of body weight intravenously every eight hours; some doses can be omitted depending on the patient's tolerance for the drug. In addition, after infusion, additional antigen or fragments containing T-cell eliciting epitope(s) may be administered to the patient to further expand the T-cell number. The antigen or epitope may be formulated with an adjuvant and/or may be in a liposomal formulation.

The cytotoxic T-cells can also be modified by introduction of a viral vector containing a DNA encoding TNF and reintroduced into a host in an effort to enhance the anti-tumor activity of the cells. Other cytokines can also be used.

The recombinant vector can be administered using any acceptable route, including, for example, scarification and injection, e.g., intradermal, subcutaneous, intramuscular, intravenous or intraperitoneal.

For parenteral administration, the recombinant vectors will typically be injected in a sterile aqueous or non-aqueous solution, suspension or emulsion in association with a pharmaceutically-acceptable carrier such as physiological saline.

REFERENCE EXAMPLE 1

CONSTRUCTION OF VECTORS

Pox Viruses

A number of pox viruses have been developed as live viral vectors for the expression of heterologous proteins (Cepko et al., *Cell* 37:1053-1062 (1984); Morin et al., *Proc. Natl. Acad. Sci. USA* 84:4626-4630 (1987); Lowe et al., *Proc. Natl. Acad. Sci. USA*, 84:3896-3900 (1987); Panicali & Paoletti, *Proc. Natl. Acad. Sci. USA*, 79:4927-4931 (1982); Mackett et al., *Proc. Natl. Acad. Sci. USA*, 79:7415-7419 (1982)). Representative fowlpox and swinepox virus are available through the ATCC under accession numbers VR-229 and VR-363, respectively.

DNA Vectors For *In Vivo* Recombination With A Parent Virus

Genes that code for desired carcinoma associated antigens are inserted into the genome of a pox virus in such a manner as to allow them to be expressed by that virus along with the expression of the normal complement of parent virus proteins. This can be accomplished by first constructing a DNA donor vector for *in vivo* recombination with a pox virus.

In general, the DNA donor vector contains the following elements:

(i) a prokaryotic origin of replication, so that the vector may be amplified in a prokaryotic host;

(ii) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector (e.g., a gene encoding antibiotic resistance);

(iii) at least one gene encoding a desired protein located adjacent to a transcriptional promoter capable of directing the expression of the gene; and

(iv) DNA sequences homologous to the region of the parent virus genome where the foreign gene(s) will be inserted, flanking the construct of element (iii).

Methods for constructing donor plasmids for the introduction of multiple foreign genes into pox virus are described in W091/19803, the techniques of which are incorporated herein by reference. In general, all DNA fragments for construction of the donor vector, including fragments containing transcriptional promoters and fragments containing sequences homologous to the region of the parent virus genome into which foreign genes are to be inserted, can be obtained from genomic DNA or cloned DNA fragments. The donor plasmids can be mono-, di-, or multivalent (i.e., can contain one or more inserted foreign gene sequences).

The donor vector preferably contains an additional gene which encodes a marker which will allow identification of recombinant viruses containing inserted foreign DNA. Several types of marker genes can be used to permit the identification and isolation of recombinant viruses. These include genes that encode antibiotic or chemical resistance (e.g., see Spyropoulos et al., *J. Virol.*, 62:1046 (1988); Falkner and Moss., *J. Virol.*, 62:1849 (1988); Franke et al., *Mol. Cell. Biol.*, 5:1918 (1985), as well as genes such as the *E. coli lac*Z gene, that permit identification of recombinant viral plaques by colorimetric assay (Panicali et al., *Gene*, 47:193-199 (1986)).

Integration Of Foreign DNA Sequences Into
<u>The Viral Genome And Isolation Of Recombinants</u>

Homologous recombination between donor plasmid DNA and viral DNA in an infected cell results in the formation of recombinant viruses that incorporate the desired elements. Appropriate host cells for *in vivo* recombination are generally eukaryotic cells that can be infected by the

virus and transfected by the plasmid vector. Examples of such cells suitable for use with a pox virus are chick embryo fibroblasts, HuTK143 (human) cells, and CV-1 and BSC-40 (both monkey kidney) cells. Infection of cells with pox virus and transfection of these cells with plasmid vectors is accomplished by techniques standard in the art (Panicali and Paoletti, U.S. Patent No. 4,603,112, WO89/03429).

Following *in vivo* recombination, recombinant viral progeny can be identified by one of several techniques. For example, if the DNA donor vector is designed to insert foreign genes into the parent virus thymidine kinase (TK) gene, viruses containing integrated DNA will be TK^- and can be selected on this basis (Mackett et al., *Proc. Natl. Acad. Sci. USA*, 79:7415 (1982)). Alternatively, co-integration of a gene encoding a marker or indicator gene with the foreign gene(s) of interest, as described above, can be used to identify recombinant progeny. One preferred indicator gene is the *E. coli lacZ* gene: recombinant viruses expressing β-galactosidase can be selected using a chromogenic substrate for the enzyme (Panicali et al., *Gene*, 47:193 (1986)).

<u>Characterizing The Viral Antigens Expressed By Recombinant Viruses</u>

Once a recombinant virus has been identified, a variety of methods can be used to assay the expression of the polypeptide encoded by the inserted gene. These methods include black plaque assay (an *in situ* enzyme immunoassay performed on viral plaques), Western blot analysis, radioimmunoprecipitation (RIPA), and enzyme immunoassay (EIA).

EXAMPLE I

Generation of PSA Specific Immune Response

Materials and Methods

Recombinant Vaccinia Virus

A 786 bp DNA fragment encoding the entire open reading frame of human prostate specific antigen was amplified by reverse transcriptase PCR (GeneAmp RNA PCR Kit, Perkin Elmer, Norwalk, CT) from total RNA extracted from the human metastatic prostate adenocarcinoma cell line, LNCaP.FGC (CRL 1740, American Type Culture Collection (ATCC), Rockville, MD). The predicted amino acid sequence derived from the PSA coding sequence was shown to be nearly identical to the published sequence (Lundwall, et al., 1987), differing only in a change from asparagine to tyrosine at position 220. The PSA DNA fragment, containing the entire coding sequence for PSA, 41 nucleotides of the 5' untranslated region, and 520 nucleotides of the 3' untranslated region, was inserted into the Xba I restriction endonuclease cleavage site of the vaccinia virus transfer vector pT116. The resulting plasmid, designated pT1001, contains the PSA gene under the control of the vaccinia virus 40K promoter (Gritz, et al. 1990) and the *E. coli lacZ* gene under the control of the fowlpox virus C1 promoter (Jenkins, et al., 1991). The foreign genes are flanked by DNA sequences from the Hind III M region of the vaccinia genome. A plaque-purified isolate from the Wyeth (New York City Board of Health) strain of vaccinia was used as the parental virus in the construction of the recombinant vaccinia virus. The generation of recombinant vaccinia virus was accomplished via homologous recombination between vaccinia sequences in the Wyeth vaccinia genome and the corresponding sequences in pT1001 in vaccinia-infected RK_{13} cells (CCL 37, ATCC) transfected with pT1001. Recombinant virus was identified using a chromogenic assay, performed on viral plaques *in situ*, that detects expression of the *lacZ* gene product in the presence of halogenated indolyl-beta-D-galactoside (Bluo-gal), as described previously (Panacali, et al., 1986). Appropriate blue recombinant viruses were purified by four rounds of plaque-purification. Virus stocks were prepared

by clarifying infected RK_{13} cell lysates followed by centrifugation through a 36% sucrose cushion.

Characterization of Recombinant Virus

Southern Analysis of DNA recombination

The recombinant vaccinia genome was analyzed by viral DNA extraction, restriction endonuclease digestion with Hind III, and Southern blotting as previously described (Kaufman *et al.*, 1991).

Western Analysis of protein expression

Confluent BSC-40 cells were infected with either parental wild type vaccinia virus (designated V-Wyeth) or recombinant vaccinia-PSA (designated rV-PSA) at an MOI of 1 in Dulbecco's Modified Eagle's Medium containing 2% fetal bovine serum. After an overnight infection, the medium was removed from the cells, and an aliquot was methanol precipitated to assay for the presence of secreted PSA. The infected cells were lysed in hypotonic lysis buffer (150 mM NaCl, 0.05% EDTA, 10 mM KCl, 1 mM PMSF) and then sonicated. Cell lysates and culture media were electrophoresed on an SDS-10% acrylamide gel. The proteins were transblotted to nitrocellulose, and the blot was incubated with a rabbit antibody specific for PSA (P0798, Sigma Chemical Co., St. Louis, MO) for 4 hours at ambient temperature, washed, and then incubated with goat anti-rabbit phosphatase-labeled secondary antibody (AP, Kirkegaard & Perry Laboratories, Gaithersburg, MD) and developed according to the manufacture's instructions.

Generation of B-cell lines

Monkey autologous B lymphoblastoid cell lines (BLCL) were established by infecting 1×10^5 freshly isolated PBMCs in 100 ml of RPMI 1640 supplemented with L-glutamine, gentamicin, and 10% FCS

(Biofluids, Rockville, MD) with 100 ml supernatant from S594 cells (kindly provided by Dr. M. D. Miller, Harvard Medical School, New England Regional Primate Research Center, Southborough, MA), which contains the baboon herpesvirus *Herpes papio*, in a 96 well, flat-bottomed plate (Costar, Cambridge, MA). Following transformation, cells were expanded, and media changed once weekly.

Immunization of Monkeys

Twelve juvenile male rhesus monkeys (*Macaca mulatta*), ages 1 to 2 years, were assigned to three vaccination groups of four animals each. One animal from each group was prostatectomized. Animals were immunized 3 times on days 1, 29, and 57. Doses of either 1×10^7 or 1×10^8 PFU of rV-PSA were administered to 4 animals by skin scarification. V-Wyeth (1×10^8 PFU) was administered to 4 animals as controls. The animals were housed and maintained at the Toxicology Research Laboratory, University of Illinois at Chicago (TRL/UIC) in accordance with the guidelines of the National Cancer Institute Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services Publication NIH 85-23, revised 1985 by the FDA Center for Biologics Evaluation and Research Office of Biological Product Review, Division of Product Quality Control, Pathology and Primatology Laboratory, Bethesda, MD).

Toxicology

Physical examinations were performed on ketamine (Ketamine® HCl, 10 mg/kg I.M.) sedated animals. Rectal temperatures and weights were recorded for each monkey on a weekly basis. The vaccination site was observed and erythema and swelling were measured by caliper. Each animal was examined for regional lymphadenopathy, hepatomegaly, and splenomegaly. Any other gross abnormalities were also recorded.

Blood was obtained by venipuncture from the femoral vein of ketamine sedated animals before and after each immunization. A complete blood count, differential, hepatic and renal chemistry evaluation was performed on each monkey by TRL/UIC. Results were compared to normal primate values (Kantor *et al.*, 1992b). Circulating levels of PSA before and after immunization were analyzed by radioimmunoassay (Tandem^{TM}, Hybritech, San Diego, CA).

Measurement of Antibody Titers

Prior to each immunization and 2 weeks following each immunization, anti-PSA antibody was quantified by ELISA. Microtiter plates were coated with purified PSA (100 ng/well, Calbiochem, La Jolla, CA), ovalbumin (100 ng/well, Sigma), or 1×10^7 PFU/well UV-inactivated V-Wyeth in PBS. The plates were blocked with 2% BSA in PBS, dried, and stored at -20° C until used. The plates were incubated with serum diluted 1:5, as well as a monoclonal antibody for PSA (DAKO M750, Denmark) as a standard control, for 24 hours at 4° C. Plates were washed several times with PBS containing 1% BSA, and incubated at 37° C for 45 min with horseradish peroxidase-conjugated goat anti-human IgG or IgM heavy chain specific antiserum (1:8000) (Southern Biotechnology Associates, Birmingham, AL) and antibody detected by HRP substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacture's instructions. The absorbance of each well was read at 405 nm using a Bio-Tek EL310 microplate ELISA reader (Winooski, VT).

Lymphoproliferative Assay

Autologous monkey BLCL were plated at a density of 3×10^6 cells/well in 24 well plates with 160 mg/well purified PSA (Fitzgerald, Concord, MA) or 160 mg/well ovalbumin (Sigma) at 37° C for 24 hours. Cells were then γ-irradiated (14000 rad), harvested, washed and

suspended at a final concentration of 1 x 10^7/ml. Fresh monkey PBMCs from heparinized blood, 6 weeks to 7 months after the last immunization, were isolated on lymphocyte separation medium (Organon Teknika, West Chester, PA). Lymphoproliferative responses were evaluated by co-culturing 1.5 x 10^5 cells with 5 x 10^5 cells/well of autologous BLCL in 0.2 ml of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum in flat-bottomed 96 well plates (Costar) for 5 days. PBMCs were cultured with 2 x 10^7 PFU/ml UV-inactivated V-Wyeth as a recall antigen or 2 mg/ml Con-A as positive controls. Cells were labeled for the final 12-18 h of the incubation with 1 mCi/well [^{3}H]thymidine (New England Nuclear, Wilmington, DE) and harvested with a PHD cell harvester (Cambridge Technology, Cambridge, MA). The incorporated radioactivity was measured by liquid scintillation counting (LS 6000IC; Beckman, Duarte, CA). The results from triplicate wells were averaged and are reported as mean ± SEM.

Results

Generation and Characterization of Recombinant Virus

The cDNA fragment encoding the open reading frame of human PSA was obtained by reverse transcriptase PCR using PSA specific oligonucleotide primers 5' TCTAGAAGCCCCAAGCTTACCACCTGCA 3' (SEQ. ID. NO.:1), 5' TCTAGATCAGGGGTTGGCCACGATGGTGTCCTTGATCCACT 3' (SEQ. ID. NO.:2), and ligated into the vaccinia virus transfer vector pT106. This vector contains a strong vaccinia virus early/late promoter (designated P40) upstream of the multiple cloning site to drive the synthesis of the inserted gene product. The ligation and orientation of the PSA DNA fragment, as well as promoter position were verified by PCR and sequencing. The chimeric vector construct was inserted into the vaccinia virus genome Hind III M site by homologous recombination as previously

reported (Kaufman, et al., (1991)), and confirmed by Southern analysis probing with ^{32}P radiolabeled DNA corresponding to PSA sequences and vaccinia sequences in the Hind III M region (data not shown). The entire cDNA sequence of PSA in the vaccinia virus clone was shown to be nearly identical to the published sequences (Lundwall, et al., 1987).

Expression of recombinant protein was confirmed by western blot analysis of supernatant fluids and protein extracts from rV-PSA infected BSC-40 cells. These cells are routinely used for the evaluation of recombinant vaccinia products (Moss, et al., 1993). Incubation of cell supernatant blots from rV-PSA infected cells with rabbit anti-PSA antibody revealed a single immunoreactive polypeptide of approximately 33,000 daltons (Figure 1, lanes 2-4). Similarly, incubation of protein extract blots from rV-PSA infected cells revealed a single band of the same molecular weight (Figure 1, lanes 7-9). This is consistent with the predicted size of the PSA molecule (Armbruster, et al., 1993; Wang, et al., 1982). Cell supernatant blots (lane 1) or protein extract blots (lane 6) from cells infected with parental strain V-Wyeth remained negative for expression of PSA. These results thus demonstrate that a recombinant vaccinia virus can faithfully express the human PSA gene product.

Rhesus Monkey Model

The prostate gland of the rhesus monkey is structurally and functionally similar to the human prostate (Wakui, et al., 1992). At the molecular level, there is 94% homology between both the amino acid and nucleic acid sequences of rhesus PSA (Gauthier, et al., 1993) and human prostate specific antigen (Karr, et al., 1995; Lundwall, et al., 1987). Human PSA is essentially an autoantigen in the rhesus monkey.

Experimental Design

Table 1 delineates the protocol used in the immunization of 12 rhesus monkeys with either rV-PSA or the control V-Wyeth by skin scarification. Three groups of 4 animals were immunized with either rV-PSA at 1×10^7 PFU/dose, rV-PSA at 1×10^8PFU/dose, or V-Wyeth at 10^8PFU/dose 3 times at 4 week intervals. These doses were chosen to ascertain the maximum tolerated dose for safety as well as to obtain maximum humoral and cell-mediated responses to PSA.

The rhesus monkeys were divided into 3 groups: high dose V-Wyeth, low dose rV-PSA, and high dose rV-PSA. One animal in each group was surgically prostatectomized to parallel two situations with regard to potential therapy in humans: (a) prostate intact, with primary and/or metastatic disease; or (b) patients prostatectomized with prostate cancer metastatic deposits. The presence of an intact prostate gland could conceivably serve as an antigen 'sink', either inducing anergy through persistence of antigen, or masking immunological effects by sequestering reactive cells or antibodies.

Physical Consequence of Immunization

The area of the lesions induced by rV-PSA or V-Wyeth was analyzed 7 days following each inoculation. In general, more induration was seen after the first inoculation, compared to the second inoculation (Figure 2A). After the third inoculation, there was no swelling of the vaccination site. The duration of the lesion following each immunization was shorter after each inoculation (Figure 2B). Regional lymph node swelling following vaccination was greater in most monkeys following the first immunization, compared to the second, or third immunization (Figure 2C). In general, no differences were seen in these parameters with the use of rV-PSA or V-Wyeth. Monkeys receiving V-Wyeth were compared with those receiving rV-PSA with respect to constitutional symptoms. Mild temperature

elevations were seen in all animals following vaccination. There was no evidence of weight loss, hepatomegaly or splenomegaly in any of the animals, and there was no differences between V-Wyeth or rV-PSA treated animals (data not shown). Animals were tested for complete blood count, differential, and hepatic and renal chemistries. Complete blood counts remained within normal limits throughout the study in both V-Wyeth and rV-PSA immunized animals (Table 2). Hepatic and renal functions were assessed prior to immunization and 12 weeks following primary immunization (Table 3). Parameters analyzed included alkaline phosphatase, blood urea nitrogen, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and creatine and creatine kinase levels. There was no significant difference between animals receiving V-Wyeth or rV-PSA. There was no detectable PSA in the circulation of any of these monkeys after any immunization (detection limit was 0.1 ng/ml). At this time, which is 54 weeks post all immunizations, no toxicities were observed in monkeys of any of the groups, including those which were prostatectomized.

PSA Specific Humoral Responses

As indicated in Table 1, monkeys 1-4 were administered V-Wyeth while monkeys 5-12 were administered rV-PSA. Sera from each of these monkeys were analyzed by ELISA for immunoreactivity to PSA or UV-inactivated V-Wyeth, and ovalbumin as control antigen. Sera obtained from monkeys prior to vaccination were negative for reactivity to PSA (Table 4, PI). Fifteen days following primary immunization, monkeys in both the 1×10^8 and 1×10^7 dose rV-PSA groups developed low titer IgM antibodies specific for PSA (titers were determined at a 1:5 serum dilution). Although other isotopes of antibody were analyzed (IgG, IgA, IgM), only IgM was induced by rV-PSA throughout the observation period of 270 days. The antibody titers decreased over the 4 weeks prior to the

next inoculation. Prior to the second vaccination on day 29, 3 of 4 animals in the $1x10^7$rV-PSA group remained positive for PSA antibody, while 4 of 4 animals remained positive in the $1x10^8$rV-PSA group. Anti-PSA antibody titers increased after the second vaccination on day 29, but remained static after the third vaccination on day 57. By 270 days after the primary immunization, all animals were negative for PSA IgM antibody. Monkeys remained negative for IgG specific for PSA throughout the observation period (data nor shown). There was no correlation between rV-PSA dose and anti-PSA IgM titer, nor was there any apparent effect of prostatectomy. All monkey sera were negative for IgG or IgM to ovalbumin at all time points; as a positive control, however, the IgG titer in all three treatment groups to vaccinia virus was greater than 1:2000 as early as 29 days after the primary immunization (data not shown).

In general, vaccinia virus is a weak human pathogen (Paoletti *et al.*, 1993). Following vaccination, local erythema, induration, low-grade fever, and regional lymphadenopathy are common. The virus replicates in the epidermal cells of the skin and the virus is usually cleared within 14 days. All monkeys, whether given V-Wyeth or rV-PSA, exhibited the usual low grade constitutional symptoms of a vaccinia virus infection (Figure 2). There was no evidence of any adverse effects as indicated by changes in blood counts, differentials, hepatic and renal chemistries (Tables 2-3). The monkeys appeared healthy, without any physical signs of toxicity, throughout the 54 weeks of observation.

Although the rV-PSA construct was unable to elicit an anti-PSA IgG response, PSA specific IgM responses were noted in all rV-PSA immunized monkeys regardless of dose level (Table 4). These antibody responses were of low titer, short lived and could not be boosted, indicating induction of a primary response but not memory B-cells or affinity maturation.

PSA Specific Lymphoproliferative Assay

PSA specific T-cell responses in monkeys immunized with rV-PSA or V-Wyeth were analyzed using a lymphoproliferative assay. As seen in Table 5, the PBMCs from all monkeys analyzed responded, regardless of whether they received rV-PSA or V-Wyeth, to the lymphocyte mitogen concanavalin-A, as well as with the recall antigen UV-inactivated V-Wyeth. Differential responses to PSA *versus* medium alone or ovalbumin were seen in 1 animal (number 6) in the 1 x 10^7 PFU rV-PSA group. All PBMCs from animals in the 1 x 10^8 PFU rV-PSA group, however, responded to PSA in this assay. This experiment was repeated 5 times with similar results and data shown in Table 5 is from PBMCs isolated from monkeys 270 days after the primary immunization. No differences in PSA specific T-cell responses were seen in the prostatectomized monkeys.

To investigate cell mediated responses to the administration of rV-PSA, lymphoproliferative assays were performed using PBMCs from animals receiving the recombinant vaccine. One of four monkeys receiving the lower dose of rV-PSA (1 x 10^7 PFU) and four of four receiving the higher dose (1 x 10^8 PFU) maintained specific T-cell responses to PSA protein up to 270 days following primary immunization as indicated by the lymphoproliferative assay (Table 5). Prostatectomy appeared to have no effect on either the humoral or cellular responses of monkeys receiving rV-PSA. Evidence of PSA specific T-cell responses in monkeys lacking mature antibody isotopes could be due to two distinct events following vaccination with rV-PSA: a T-cell independent event, leading to IgM production, and a T-cell dependent event, leading to specific lymphoproliferative responses.

Table 1 Inoculation protocol of rhesus monkeys with the PSA recombinant and wild-type vaccinia virus

Monkey	Prostate	Immunogen	Dose* (PFU)
1	Yes	V-Wyeth	$1x10^8$
2	Yes	V-Wyeth	$1x10^8$
3	Yes	V-Wyeth	$1x10^8$
4	No	V-Wyeth	$1x10^8$
5	Yes	rV-PSA	$1x10^7$
6	Yes	rV-PSA	$1x10^7$
7	Yes	rV-PSA	$1x10^7$
8	No	rV-PSA	$1x10^7$
9	Yes	rV-PSA	$1x10^8$
10	Yes	rV-PSA	$1x10^8$
11	Yes	rV-PSA	$1x10^8$
12	No	rV-PSA	$1x10^8$

* All animals received 3 immunizations at 4 week intervals.

Table 2
Mean WBC count, hematocrit, and differential count in rhesus monkeys receiving recombinant or wild-type vaccine

Test	Normal ranges	V-Wyeth (n=4)		rV-PSA (n=8)	
		Before immunization[a]	After immunization[b]	Before immunization	After immunization
WBC	7-15 x 10^3	5.0 ± 0.8	5.1 ± 0.5	5.2 ± 0.7	5.8 ± 0.9
Hematocrit (vol. %)	33-43	37.4 ± 0.2	37.0 ± 0.1	37.8 ± 0.4	37.0 ± 0.5
Lymphocytes	1-7 x 10^3	2.8 ± 0.7	3.9 ± 0.5	2.2 ± 0.4	3.5 ± 0.8
SEGS[c] (%)	3-69	2.0 ± 0.2	0.78 ± 0.2	2.9 ± 0.6	1.9 ± 0.3
Monocytes (%)	0-8	0.1 ± 0.05	0.2 ± 0.04	0.1 ± 0.04	0.2 ± 0.50
Eosinophils (%)	0-8	0.1 ± 0.02	0.2 ± 0.10	0.1 ± 0.03	0.1 ± 0.02

a 1 week prior to primary immunization
b 12 weeks following primary immunization
c Segmented lymphocytes

Table 3 Mean serum chemistry values in rhesus monkeys receiving recombinant or wild-type vaccine

Test	Normal ranges	V-Wyeth (n=4)		rV-PSA (n=8)	
		Before immunization[a]	After immunization[b]	Before immunization	After immunization
ALKP[c] (u/l)	200-800	451 ± 48	610 ± 33	339± 74	454 ± 47
BUN[d] (mg/dl)	12-30	19.0 ± 3.0	17.8 ± 0.9	17.1± 0.6	20.5± 1.0
ALT[e] (u/l)	20-60	25.2 ± 1.9	22.8 ± 1.0	28.9± 5.3	25.8 ± 1.6
AST[f] (u/l)	40-80	37.8 ± 2.3	31.8 ± 4.4	37.9 ± 3.6	31.9 ± 2.4
LDH[g] (u/l)	200-500	194 ± 20	212 ± 21	236 ± 41	194 ± 13
Creatine (mg/dl)	0.5-1.0	0.9 ± 0.10	0.8 ± 0.03	0.8 ± 0.05	0.8 ± 0.02
Creatine Kinase (u/l)	500-2000	662 ± 112	466± 119	498 ± 120	563 ± 81

a 1 week prior to primary immunization

b 12 weeks following primary immunization

c Alkaline phosphatase

d Blood urea nitrogen

e Alanine aminotransferase

f Aspartate aminotransferase

g Lactate dehydrogenase

Table 4 Primate IgM[a] Response to Inoculation with rV-PSA

Monkey	Immunogen	Dose (PFU)	Days Post Immunization[b]						
			PI[d]	15	29[e]	43	57[e]	71	270
1	V-Wyeth	$1x10^8$	ND[f]	ND	ND	ND	ND	ND	ND
2	V-Wyeth	$1x10^8$	ND	ND	ND	ND	ND	ND	ND
3	V-Wyeth	$1x10^8$	ND	ND	ND	ND	ND	ND	ND
4[c]	V-Wyeth	$1x10^8$	ND	ND	ND	ND	ND	ND	ND
5	rV-PSA	$1x10^7$	ND	>40	5	20	>40	>40	ND
6	rV-PSA	$1x10^7$	ND	>40	ND	ND	20	20	ND
7	rV-PSA	$1x10^7$	ND	>40	5	20	20	20	ND
8[c]	rV-PSA	$1x10^7$	ND	>40	5	10	>40	>40	ND
9	rV-PSA	$1x10^8$	ND	20	5	20	10	10	ND
10	rV-PSA	$1x10^8$	ND	20	5	40	>40	NT[g]	ND
11	rV-PSA	$1x10^8$	ND	>40	>40	>40	>40	>40	ND
12[c]	rV-PSA	$1x10^8$	ND	>40	20	40	20	20	ND

[a] All monkey seras were negative for IgG to PSA at all time points; All seras were positive for IgG to vaccinia virus (>1:2000) at day 71.

[b] Monkeys received vaccinations on days 1, 29, and 57. Sera (1:5) was tested by ELISA. Titers were calculated using an O.D. of 0.4.

[c] Animal was prostatectomized.

[d] PI, Pre-immune.

[e] Animals bled before boosting.

[f] ND, not detectable; limit of detection was <1:5 dilution.

[g] NT, not tested.

Table 5 PSA Specific Lymphoproliferative T-cell Responses of Rhesus PBMCs 270 Days Following Inoculation with rV-PSA

Monkey	Immunogen	Dose (PFU)	Antigen[a] Medium	Con A	Oval	UV-Wyeth	PSA[d]
1	V-Wyeth	$1x10^8$	397	65701	376	24785	414
2[b]	V-Wyeth	$1x10^8$	NT	NT	NT	NT	NT
3	V-Wyeth	$1x10^8$	450	84860	522	18859	413
4[c]	V-Wyeth	$1x10^8$	532	107840	553	16571	387
5	rV-PSA	$1x10^7$	412	85276	408	6040	539
6	rV-PSA	$1x10^7$	401	96398	404	7776	**3,134**
7	rV-PSA	$1x10^7$	417	90801	522	10908	434
8[c]	rV-PSA	$1x10^7$	1069	99216	744	15346	484
9	rV-PSA	$1x10^8$	384	106248	386	14499	**10,635**
10	rV-PSA	$1x10^8$	432	92263	404	19872	**18,561**
11	rV-PSA	$1x10^8$	411	94055	1063	5124	**16,245**
12[c]	rV-PSA	$1x10^8$	420	124896	392	11944	**12,945**

[a] Antigen concentrations were: Con a (2 μg/ml); Ovalbumin (100 μg/ml); UV-Wyeth ($2x10^7$ pfu/ml); and PSA (100 μg/ml). Each value represents a mean CPM of triplicate samples. Standard deviation never exceeded 10%.

[b] NT, Not Tested. B-cells were not transformed for this animal.

[c] Animal was prostatectomized.

[d] Values in bold are significant when compared to their respective medium control values ($p < 0.001$).

EXAMPLE II

Identification Of Potential Prostate Specific Antigen (PSA) Specific T Cell Epitopes

Since the entire amino acid sequence of human PSA is known and human class 1 HLA A2 consensus motifs have been described, studies were undertaken to identify a series of peptides that would potentially bind class 1 A2 molecules. A2 was chosen since it is the most common HLA class 1 molecule being represented in approximately 50% of North American Caucasians and 34% of African Americans. The peptide sequence of PSA was thus examined for matches to the consensus motifs for HLA A2 binding peptides. Peptides were only selected if their sequence diverged sufficiently from the PSA-related human glandular kallikrein (HGK) gene and pancreatic kallikrein antigen (PKA) sequences.

The amino acid sequence of human PSA was scanned using a predictive algorithm that combines a search for anchor residues with numerical assignments to all residues at all positions. The T2 cell binding assay was then used to determine which peptides bound human HLA A2 molecules. As can be seen in Table 6, PSA peptides 141-150, 154-163 and 146-154 scored positive in this assay (*Nijman, H.W., et al., Eur. J. Immunol.* 23:1215-1219, 1993). Table 7 gives the amino acid sequence of these peptides and compares them to corresponding sequences of HGK and PKA.

Table 6
PSA peptide binding assay

Antigen	MAb A2, 69
None	127.25[a]
PSA 141-150	230.34
PSA 146-154	223.97
PSA 154-163	182.30

Peptides were used at a concentration of 50μg/ml
[a]Mean channel fluorescent intensity.
CIRA2 cell line was used as positive control for anti-A2 staining [99.4 (241.15)].

Table 7
PSA peptide amino acid sequence

PSA	141-150	F	L	T	P	K	K	L	Q	C	V
HGK		-	-	R	-	R	S	-	-	-	-
PKA		-	**S**	F	-	D	D	-	-	-	-
PSA	146-154	K	L	Q	C	V	D	L	H	V	
HGK		S	-	-	-	-	S	-	-	L	
PKA		D	-	-	-	-	-	-	K	I	
PSA	154-163	V	I	S	N	D	V	C	A	Q	V
HGK		L	L	-	-	-	M	-	-	R	**A**
PKA		I	L	P	-	-	E	-	E	K	**A**

EXAMPLE III
Establishment of Human T Cell Lines
Cytolytic For Human Tumor Cells Expressing PSA

PBMC from normal healthy donors expressing the HLA A2 class 1 allele were used in an attempt to determine if PSA specific peptides are immunogenic for humans. Peptides 141-150 and 154-163 were used in this study. The methodology used for the establishment of these cell lines involves pulsing of PBMC with peptide and IL-2 as previously described (*Tsang, K.Y., et al. JNCI, in press* and in U.S. Application Serial No. 08/396,385, the disclosure of which is herein incorporated by reference). T cell lines were able to be established from 5/6 normal donors using PSA peptide 141-150 and from 6/6 normal donors using PSA peptide 154-163. Moreover, PBMC were obtained from two prostate cancer patients. T cell lines were established from these PBMC cultures using peptide 154-163.

Some of these T cell lines have been phenotyped. As seen in Table 8, one cell line designated T-866, which was derived from pulsing with peptide 141-150, contains appreciable amounts of CD4+/CD8+ double positive cells and another cell line, designated T-1538, derived from pulsing with peptide 154-163, shows a similar phenotype.

Four of the T cell lines derived from three different individuals were then assayed for their ability to lyse human cells (Table 9). As seen in Table 9, the T cell line designated T-866, derived from peptide 141-150, was able to lyse T2 cells when pulsed with the appropriate peptide (141-150). No lysis was seen using the PSA negative human colon cancer cell line COLO-205. While 80% lysis was seen using the LNCAP PSA containing human prostate cancer cell line. When employing the NK target K562, which measures non-specific lysis due to NK cell activity, only 23% lysis was obtained. Similar results were seen employing a different T cell

line obtained from the same patient which was derived from pulsing with PSA peptide 154-163. Two additional T cell lines which were derived from peptide 154-163 were also analyzed. One was from a normal donor (T-1538) and one was from a prostate cancer patient (T-PC2). As can be seen in Table 9, employing both of these T cell lines, enhanced lysis was seen when the T2 cell line was pulsed with the 154-163 peptide and enhanced lysis was seen when employing the PSA expressing prostate specific cell line LNCAP, as compared to COLO-205 or K562. These studies demonstrate that T cell lines can be established using the peptides and protocols generated here which have the ability to lyse PSA expressing human prostate carcinoma cells.

Table 8
Flow cytometry analysis of PSA peptide specific T cells

T-cell Line	PSA Peptide	CD3	CD4	CD8	CD4/CD8	CD56
T-866	141-150	96	35	6.5	59	0
T-1538	154-163	94	5.2	32	62	0

Table 9
Cytotoxic effects of PSA peptide specific T cells

T-cell Line	PSA Peptide	% specific release (lysis) T2	T2+ peptide	LNCAP	K562	COLO-205
T-866	141-150	10[a]	40	80	23	7
T-866	154-160	16	35	60	22	10
T-1538	154-160	10	40	29	3	10
T-PC2	154-160	15	35	35	2	8

[a]Percent of ^{111}In specific release
24 hour cytotoxic assay (E:T ratio, 25:1) (SD<2.5%)

The following is a listing of publications referred to in the foregoing specification.

Armbruster, D. A. Prostate-specific antigen: biochemistry, analytical methods, and clinical application. *Clinical Chemistry*, **39**:181-195, (1993).

Bilhartz, D. L., Tindall, D. J., and Oesterling, J. E. Prostate-specific antigen and prostatic acid phosphatase: biomolecular and physiological characteristics. *Urology*, **38**:95-102, (1991).

Brawer, M. K., and Lange, P. H. Prostate-specific antigen and premalignant change: implications for early detection. *CA Cancer Journal Clinic*, **39**:361-375, (1989).

Carter, H. B., and Coffey, D. S. The prostate: an increasing medical problem. *Prostate*, **16**:39-48, (1990).

Chatterjee, M. B., Foon, K. A., and Kohler, H. Idiotypic antibody immunotherapy of cancer. *Cancer Immunology and Immunotherapy*, **38**:75-82, (1994).

Cheever, M. A., Chen, W., Disis, M. T., and Peace, D. J. T-cell immunity to oncogenic proteins including mutated RAS and chimeric BCR-ABL. *Annals of the New York Academy of Science*, **690**:101-112, (1993).

Choe, B. K., Frost, P., Morrison, M. K., and Rose, N R. Natural killer cell activity of prostatic cancer patients. *Cancer Investigations*, **5**:285-291, (1987).

Conry, R. M., Salch, M. N., Schlom, J., and LoBuglio, A. F. Breaking tolerance to carcinoembryonic antigen with a recombinant vaccinia virus vaccine in man. *American Association of Cancer Research* (Abstract), (1994).

Correale, P., Zaremba, S., Nieroda, C., Zhu, M. Z., Schmitz, J., Schlom, J., and Tsang, K. Y. In vitro stimulation of human cytotoxic T lymphocytes specific for peptides derived from prostate specific antigen. *9th International Congress of Immunology* (Abstract), (1995).

Disis, M. L., Smith, J. W., Murphy, A. A., Chen, W., and Cheever, M. A. In vitro generation of human cytolytic T-cells specific for peptides from the HER-2/neu protooncogene protein. *Cancer Research*, **54**:1071-1076, (1994).

Donovan, J. F., Lubaroff, D. M., and Williams, R. D. Immunotherapy of prostate cancer. *Problems in Urology*, **4**:489-505, (1990).

Foon, K. A., Chakraborty, M., John, W., Sherratt, A., Kohler, H., and Bhattacharya-Chatterjee, M. Active immunity to the carcinoembryonic antigen (CEA) in patients treated with an anti-idiotype monoclonal antibody vaccine. *Society for Biological Therapy* (Abstract), (1994).

Gauthier, E. R., Chapdelaine, P., Tremblay, R. R., and Dube, J. Y. Characterization of rhesus monkey prostate specific antigen cDNA. *Biochimica Biophysica Acta*, **1174**:207-210, (1993).

Gritz, L., Destree, A., Cormier, N., Day, E., Stallard, V., Caiazzo, T., Mazzara, G., and Panicali, D. Generation of hybrid genes and proteins by vaccinia virus-mediated recombination: application to human immunodeficiency virus type 1 env. *J. Virol.* 64:5948-5957, (1990).

Helling, F., and Livingston, P. O. Ganglioside conjugate vaccines. Immunotherapy against tumors of neuroectodermal origen. *Molecular and Chemical Neuropathology*, **21**:299-309, (1994).

Helling, F., Calves, M., Shang, Y., Oettgen, H. F., and Livingston, P. O. Construction of immunogenic GD3-conjugate vaccines. *Annals of the New York Academy of Science*, **690**:396-397, (1993).

Huang, C, L., Brassil, D., Rozzell, M., Schellhammer, P. F., and Wright, G. L. Comparison of prostate secretory protein with prostate specific antigen and prostatic acid phosphatase as a serum biomarker for diagnosis and monitoring patients with prostate carcinoma. *Prostate*, **23**:201-212, (1993).

Ioannides, C. G., Fisk, B., Fan, D., Biddison, W. E., Wharton, J. T., and O'Brian, C. Cytotoxic T cells isolated from ovarian malignant ascites recognize a peptide derived from the HER-2/neu proto-oncogene. *Cellular Immunology*, **151**:225-234, (1993).

Irvine, K., Kantor, J., and Schlom, J. Comparison of a CEA-recombinant vaccinia virus, purified CEA, and an anti-idiotype antibody bearing the image of a CEA epitope in the treatment and prevention of CEA-expressing tumors. *Vaccine Research*, **2**:79-94, (1993).

Isaacs, J. T., Feitz, W. F., and Scheres, J. Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostatic cancers. *Prostate*, **9**:261-281, (1986).

Jenkins, S., Gritz, L., Fedor, C., O'Neil, E., Cohen, L. and Panicali, D. Formation of lentivirus particles in mammalian cells infected with recombinant fowlpox virus. *AIDS Research and Human Retroviruses* 7:991-998, (1991).

Kantor, J., Irvine, K., Abrams, S., Kaufman, H., Dipietro, J., and Schlom, J. Antitumor activity and immune responses induced by a recombinant carcinoembryonic antigen-vaccinia virus vaccine. *Journal of the National Cancer Institute*, **84**:1084-1091, (1992a).

Kantor, J., Irvine, K., Abrams, S., Snoy, P., Olsen, R., Greiner, J., Kaufman, H., Eggensperger, D., and Shlom, J. Immunogenicity and safety of a recombinant vaccinia virus vaccine expressing the carcinoembryonic antigen gene in a nonhuman primate. *Cancer Research*, **52**:6917-6925, (1992b).

Karr, J. F., Kantor, J. A., Hand, P. H., Eggensperger, D. L., and Schlom, J. Conservation pf the prostate specific antigen (PSA) gene in primates and the expression of recombinant human PSA in a transfected murine cell line. *Cancer Research*:Submitted for Publication, (1995).

Kaufman, H., Schlom, J., and Kantor, J. A recombinant vaccinia virus expressing human carcinoembryonic antigen (CEA). *International Journal of Cancer*, **48**:900-907, (1991).

Kleer, E., and Oesterling, J. E. PSA and staging of localized prostate cancer. *Urologic Clinics of North America*, **20**:695-704, (1993).

Lilja, H. Structure, function, and regulation of the enzyme activity of prostate-specific antigen. *World Journal of Urology*, **11**:188-191, (1993).

Livingston, P. O., Calves, M. J., Helling, F., Zollinger, W. D., Blake, M. S., and Lowell, G. H. GD3/proteosome vaccines induce consistent IgM antibodies against the ganglioside GD3. *Vaccine*, **12**:1199-1204, (1993).

Lundwall, A., and Lilja, H. Molecular cloning of human prostate specific antigen cDNA. *FEBS Letters*, **214**:317-322, (1987).

McEntee, M., Isaacs, W., and Smith, C. Adenocarcinoma of the canine prostate: immunohistochemical examination for secretory antigens. *Prostate*, **11**:163-170, (1987).

Moss, B. Generation of recombinant vaccinia viruses. *Current Protocols in Molecular Biology*, 2:16.15.1-16.18.9, (1993).

Oesterling, J. E. Prostate specific antigen: a critical assessment of the most useful tumor marker for adenocarcinoma of the prostate. *Journal of Urology*, **145**:907-923, (1991).

Panicali, D., Grzelecki, A. and Huang, C. Vaccinia virus vectors utilizing the β-galactosidase assay for rapid selection of recombinant viruses and measurement of gene expression. *Gene* 47:193-199, (1986).

Paoletti, E., Tartaglia, J., and Cox, W. I. Immunotherapeutic stratagies for cancer using poxvirus vectors. *Annals of the New York Academy of Sciences*, **690**:292-300, (1993).

Peace, D. J., Xue, B., Sosman, J. A., and Zhang, Y. In vitro immunization of human cytotoxic T lymphocytes specific for peptides derived from prostate specific antigen. *Cancer Vaccines: Structural Basis for Vaccine Development* (Abstract), (1994).

Powrie, F., and Coffman, R. L. Cytokine regulation of T-cell function: potential for therapeutic intervention. *Immunology Today*, **14**:270-274, (1993).

Ravindranath, M. H., Brazeau, S. M., and Morton, D. L. Efficacy of tumor cell vaccine after incorporating monophosphoryl A (MPL) in tumor cell membranes containing tumor associated ganglioside. *Experimentia*, **50**:648-653, (1994).

Ritter, G., Boosfeld, E., Adluri, R., Calves, M., Oettgen, H. F., Old, L. J., and Livingston, P. Antibody response to immunization with ganglioside GD3 and GD3 congeners (lactones, amide, and ganglisidol) in patients with malignant melanoma. *International Journal of Cancer*, **48**:379-385, (1991).

Schellhammer, P. F., and Wright, G. L. Biomolecular and clinical characteristics of PSA and other candidate prostate tumor markers. *Urologic Clinics of North America*, **20**:597-606, (1993).

Schlom, J., Kantor, J., Abrams, S., Tsang, K. Y., Panicali, D., and Hamilton, J. M. Strategies for the development of recombinant vaccines for the immunotherapy of breast cancer. *Breast Cancer Research and Treatment*, In Press.

Schroder, F. H. Experimental Models in the study of prostate cancer. Prostate Cancer. In: *International Perspectives in Urology.*, **3**:343-377, (1982).

Tsang, K. Y., Nieroda, C. A., De Filippi, R., Chung, Y. K., Yamaue, H., Greiner, J. W., and Schlom, J. Induction of human cytotoxic T cell lines directed against point-mutated p21 Ras-derived synthetic peptides. *Vaccine Research*, **3**:183-193, (1994).

Wakui, S., Furusato, M., Nomura, Y., Asari, M., and Kano, Y. Lectin histochemical study of the prostate gland of the rhesus monkey (Macaca mulatta). *Journal of Anatomy*, **181**:127-131, (1992).

Wang, M. C., Kuriyama, M., Papsidero, L. D., Loor, R. M., Valenzuela, L. A., Murphy, G. P., and Chu, T. M. Prostate antigen of human cancer patients. *Methods in Cancer Research*, **19**:179-197, (1982).

Wang, M. C., Valenzuela, L. A., Murphy, G. P., and Chu, T. M. Purification of a human prostate specific antigen. *Investigations in Urology*, **17**:159-163, (1979).

Zietman, A. L., Shipley, W. L., and Willett, C. G. Residual disease after radical surgery or radiation therapy for prostate cancer. Clinical significance and therapeutic implications. *Cancer*, **71**:959-969, (1993).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SCHLOM, JEFFREY
PANICALI, DENNIS

(ii) TITLE OF INVENTION: GENERATION OF IMMUNE RESPONSES TO
PROSTATE-SPECIFIC ANTIGEN (PSA)

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SEWALL P. BRONSTEIN; DIKE, BRONSTEIN,
ROBERTS & CUSHMAN
(B) STREET: 130 WATER STREET
(C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: US
(F) ZIP: 02129

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: RESNICK, DAVID S.
(B) REGISTRATION NUMBER: 34,235
(C) REFERENCE/DOCKET NUMBER: 44981

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 523-3400
(B) TELEFAX: (617) 523-6440

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTAGAAGCC CCAAGCTTAC CACCTGCA 28

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 41 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: unknown
  (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTAGATCAG GGGTTGGCCA CGATGGTGTC CTTGATCCAC T 41

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

What is claimed is:

1. A method for generating an immune response to prostate-specific antigen (PSA) comprising, introducing a sufficient amount of a first pox virus vector to a host to stimulate an immune response, wherein the pox virus vector has at least one insertion site containing a DNA segment encoding PSA operably linked to a promoter capable of expression in the host.

2. The method of claim 1, further comprising at at least one periodic interval after introduction of the first pox virus vector contacting the host with additional PSA or a cytotoxic T-cell eliciting epitope thereof.

3. The method of claim 2, wherein the host is contacted with the additional PSA by introducing a second pox virus vector to the host having at least one insertion site containing a DNA segment encoding the PSA operably linked to a promoter capable of expression in the host.

4. A method for generating an immune response to prostate-specific antigen (PSA) in a host, comprising:

a. contacting the host with a sufficient amount of PSA or a cytotoxic T-cell eliciting epitope thereof; and

b. at least one periodic interval thereafter contacting the host with additional PSA or a cytotoxic T-cell eliciting epitope thereof.

5. The method of claim 4, wherein the host is contacted with the additional PSA by introducing a pox virus vector to the host having at least one insertion site containing a DNA segment encoding PSA or a cytotoxic T-cell eliciting epitope thereof operably linked to a promoter capable of expression in the host.

6. The method of claim 1 or 5, wherein the pox virus is selected from the group of pox viruses consisting of suipox, avipox, capripox and orthopox virus.

7. The method of claim 6, wherein the orthopox virus is vaccinia.

8. The method of claim 7, wherein the avipox is fowlpox, canary pox and pigeon pox.

9. The method of claim 8, wherein the suipox is swinepox.

10. The method of claim 3, wherein the first pox virus vector is vaccinia and the second pox virus vector is selected from the group of pox viruses consisting of suipox, avipox, capripox and orthopox virus.

11. The method of claim 2 or 4, wherein the PSA or T-cell eliciting epitope is formulated with an adjuvant or is in a liposomal formulation.

12. The method of claim 11, wherein the adjuvant is selected from the group consisting of RIBI Detox, QS21 and incomplete Freund's adjuvant.

13. A method for generating an immune response to PSA comprising contacting a host with a cytotoxic T-cell eliciting epitope of PSA.

14. The method of claim 13, wherein the T-cell eliciting epitope is formulated with an adjuvant or is in a liposomal formulation.

15. The method of claim 12, wherein the adjuvant is selected from the group consisting of RIBI Detox, QS21 and incomplete Freund's adjuvant.

16. A pharmaceutical composition comprising a pox virus vector having at least one insertion site containing a DNA reagent encoding PSA operably linked to a promoter and a pharmaceutical carrier.

ABSTRACT

We have discovered that by using a recombinant viral vector, preferably a pox virus vector having at least one insertion site containing a DNA segment encoding prostate-specific antigen (PSA), operably linked to a promoter capable of expression in the host, a specific humoral and cellular immune response to PSA can be generated. The method preferably comprises introducing a sufficient amount of the recombinant pox virus vector into a host to stimulate the immune response, and contacting the host with additional PSA at periodic intervals thereafter. The additional PSA may be added by using a second pox virus vector from a different pox genus. In another embodiment, additional PSA can be added by contacting the host with PSA by a variety of other methods, including in one preferred embodiment adding PSA. The PSA may be formulated with an adjuvant or in a liposomal formulation.

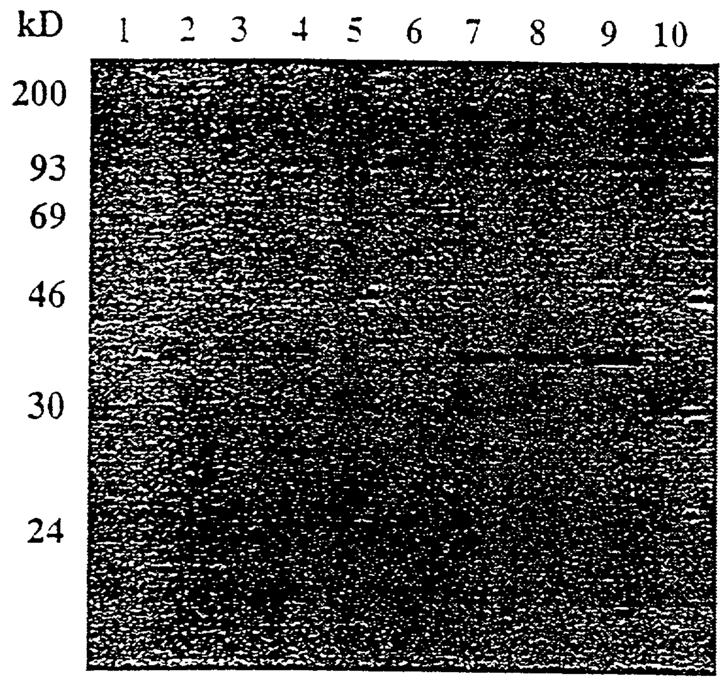


Fig 1.

Fig 2A

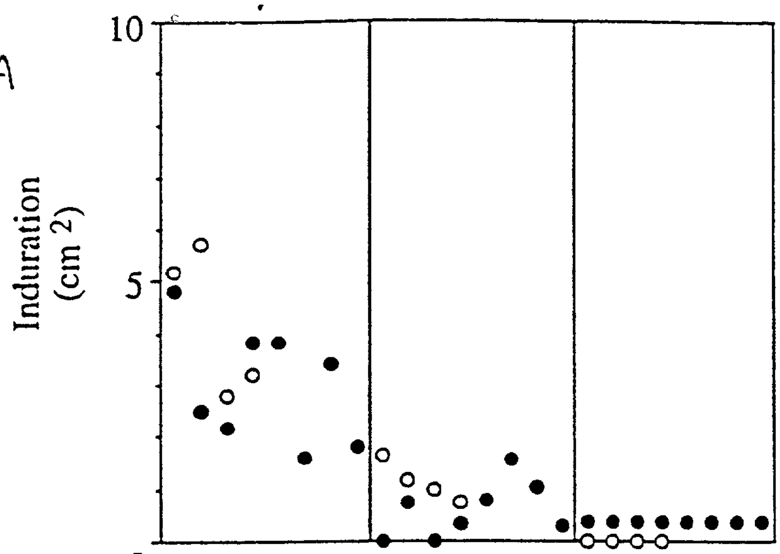


Fig 2B

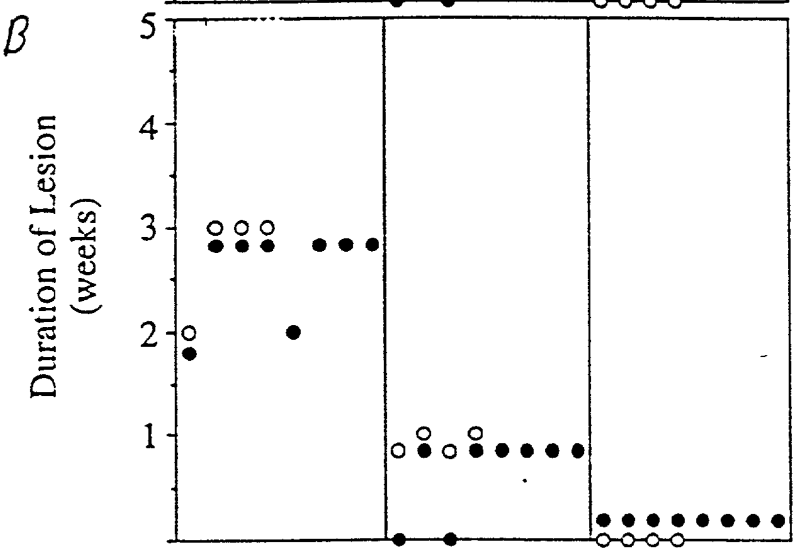
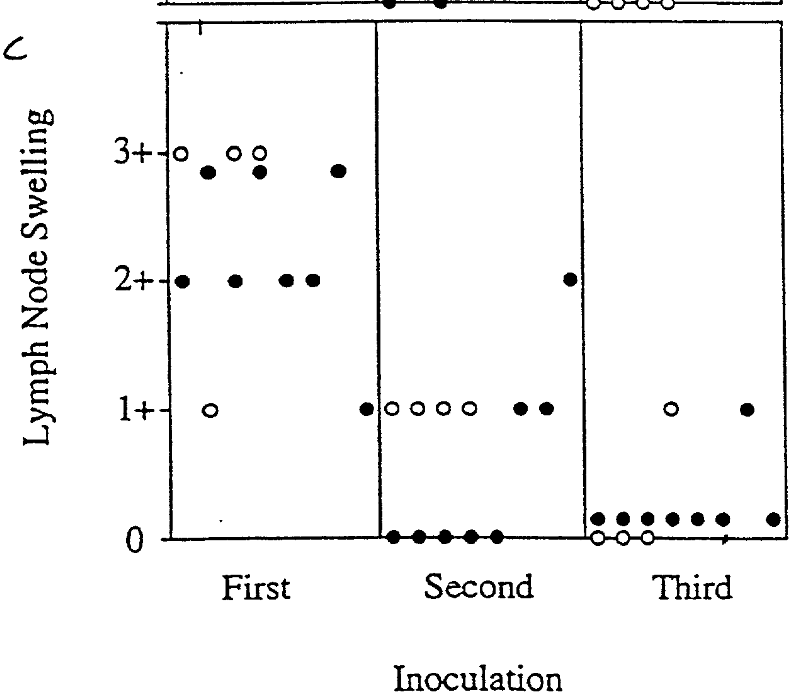


Fig 2C



DIKE, BRONSTEIN, ROBERTS & CUSHMAN
130 Water Street
Boston
Massachusetts 02109

DECLARATION AND POWER OF ATTORNEY
Original Application

ATTORNEY'S DOCKET NO (If Any)
45394

COPY

As a below named inventor, I hereby declare that
My residence, post office address and citizenship are as stated below next to my name.
I believe I am the original, first and sole inventor (if only one name is listed at 201 below) or an original, first and joint inventor (if plural names are listed at 201-203 below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

GENERATION OF IMMUNE RESPONSES TO PROSTATE SPECIFIC ANTIGEN (PSA)

which is described and claimed in:
☐ the attached specification or   xx the specification in application Serial No. 08/500,306 filed July 10, 1995
(for declaration not accompanying application)
And was amended on .... if applicable

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed

FOREIGN APPLICATION(S), IF ANY, FILED WITHIN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION			
COUNTRY	APPLICATION	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
			YES NO
			YES NO
ALL FOREIGN APPLICATIONS, IF ANY, FILED MORE THAN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION			

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No)	(Filing Date)	(Status) (Patented, pending, abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Sewall P. Bronstein	(Reg. No. 16,919)	Ernest V. Linek	(Reg. No. 29,822)	Peter J. Manus	(Reg. No. 26,766)	Brian L. Michaelis	(Reg. No. 34,221)
Donald Brown	(Reg. No. 20,845)	Linda M. Buckley	(Reg. No. 31,003)	David S. Resnick	(Reg. No. 34,235)	Cara Z. Lowen	(Reg. No. 38,227)
David G. Conlin	(Reg. No. 27,026)	Ronald I. Eisenstein	(Reg. No. 30,628)	Peter F. Corless	(Reg. No. 33,860)	John L. Welch	(Reg. No. 28,129)
George W. Neuner	(Reg. No. 26,964)	Henry D. Pahl, Jr.	(Reg. No. 20,438)	Kevin J. Fournier	(Reg. No. 34,333)	Milton Mck. Oliver	(Reg. No. 28,333)

SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO: (name and telephone number)
Sewall P. Bronstein, Esq. Dike, Bronstein, Roberts & Cushman 130 Water Street Boston, Massachusetts 02109	David S. Resnick 617/523-3400

201	FULL NAME OF INVENTOR	LAST NAME: SCHLOM	FIRST NAME: Jeffrey	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Potomac	STATE OR FOREIGN COUNTRY: Maryland	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 10301 Sorrel Ave.	CITY: Potomac	STATE OR COUNTRY: Maryland	ZIP CODE: 20854
202	FULL NAME OF INVENTOR	LAST NAME: PANICALI	FIRST NAME: Dennis	MIDDLE NAME: L.	
	RESIDENCE & CITIZENSHIP	CITY: Acton	STATE OR FOREIGN COUNTRY: Massachusetts	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 114 Nonset Path	CITY: Acton	STATE OR COUNTRY: Massachusetts	ZIP CODE: 01720
203	FULL NAME OF INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY	ZIP CODE

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE	DATE 10-25-95	DATE

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

DIKE, BRONSTEIN, ROBERTS & CUSHMAN
130 Water Street
Boston
Massachusetts 02109

DECLARATION AND POWER OF ATTORNEY
Original Application

ATTORNEY'S DOCKET NO (If Any) 45394

COPY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed at 201 below) or an original, first and joint inventor (if plural names are listed at 201-203 below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

GENERATION OF IMMUNE RESPONSES TO PROSTATE SPECIFIC ANTIGEN (PSA)

which is described and claimed in:

[ ] the attached specification or   xx the specification in application Serial No. 08/500,306 filed July 10, 1995
(for declaration not accompanying application)
And was amended on .... if applicable

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

FOREIGN APPLICATION(S), IF ANY, FILED WITHIN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION			
COUNTRY	APPLICATION	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
			YES NO
			YES NO
ALL FOREIGN APPLICATIONS, IF ANY, FILED MORE THAN 12 MONTHS PRIOR TO THE FILING DATE OF THIS APPLICATION			

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

(Application Serial No.)	(Filing Date)	(Status) (Patented, pending, abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) with full powers of association, substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Sewall P. Bronstein (Reg. No. 16,919)	Ernest V. Linek (Reg. No. 29,822)	Peter J. Manus (Reg. No. 26,766)	Brian L. Michaelis (Reg. No. 34,221)
Donald Brown (Reg. No. 20,845)	Linda M. Buckley (Reg. No. 31,003)	David S. Resnick (Reg. No. 34,235)	Cara Z. Lowen (Reg. No. 38,227)
David G. Conlin (Reg. No. 27,026)	Ronald I. Eisenstein (Reg. No. 30,628)	Peter F. Corless (Reg. No. 33,860)	John L. Welch (Reg. No. 28,129)
George W. Neuner (Reg. No. 26,964)	Henry D. Pahl, Jr. (Reg. No. 20,438)	Kevin J. Fournier (Reg. No. 34,333)	Milton McK. Oliver (Reg. No. 28,333)

SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO: (name and telephone number)
Sewall P. Bronstein, Esq. Dike, Bronstein, Roberts & Cushman 130 Water Street Boston, Massachusetts 02109	David S. Resnick 617/523-3400

201	FULL NAME OF INVENTOR	LAST NAME: SCHLOM	FIRST NAME: Jeffrey	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Potomac	STATE OR FOREIGN COUNTRY: Maryland	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 10301 Sorrel Ave.	CITY: Potomac	STATE OR COUNTRY: Maryland	ZIP CODE: 20854
202	FULL NAME OF INVENTOR	LAST NAME: PANICALI	FIRST NAME: Dennis	MIDDLE NAME: L.	
	RESIDENCE & CITIZENSHIP	CITY: Acton	STATE OR FOREIGN COUNTRY: Massachusetts	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 114 Nonset Path	CITY: Acton	STATE OR COUNTRY: Massachusetts	ZIP CODE: 01720
203	FULL NAME OF INVENTOR	LAST NAME:	FIRST NAME:	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY:	STATE OR FOREIGN COUNTRY:	COUNTRY OF CITIZENSHIP:	
	POST OFFICE ADDRESS	POST OFFICE ADDRESS:	CITY:	STATE OR COUNTRY:	ZIP CODE:

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
Jeffrey Schlom		
DATE 11/20/95	DATE	DATE

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

Practitioner's Docket No. 700953-45394-CPA-2

PATENT

JC813 U.S. PTO 09/693121 10/20/00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Schlom et al.
Application No.:	Continuation of 08/500,306
Filed:	July 10, 1995
For:	GENERATION OF IMMUNE RESPONSES TO PROSTATE-SPECIFIC ANTIGEN (PSA)

Assistant Commissioner for Patents
Washington, D.C. 20231

CHANGE OF ATTORNEY'S ADDRESS IN APPLICATION

Please send all correspondence for this application as follows:

Ronald I. Eisenstein
NIXON PEABODY LLP
101 Federal Street
Boston, MA 02110

Please direct telephone calls to:
(617) 345-6054

SIGNATURE OF PRACTITIONER

Reg. No. 34,235
Tel. No.: (617) 345-6057

David Resnick
NIXON PEABODY LLP
101 Federal Street
Boston, MA 02110

CERTIFICATE OF MAILING/TRANSMISSION (37 C.F.R. 1.8(a))

I hereby certify that, on the date shown below, this correspondence is being:

MAILING

0 deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

FACSIMILE

0 transmitted by facsimile to the Patent and Trademark Office.

Patricia Turner
Signature

Date: 10/20/00

Patricia Turner

(Change of Attorney's Address in Application,page 1 of 1)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SCHLOM, JEFFREY
PANICALI, DENNIS

(ii) TITLE OF INVENTION: GENERATION OF IMMUNE RESPONSES TO PROSTATE-SPECIFIC ANTIGEN (PSA)

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SEWALL P. BRONSTEIN; DIKE, BRONSTEIN, ROBERTS & CUSHMAN
(B) STREET: 130 WATER STREET
(C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: US
(F) ZIP: 02129

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: RESNICK, DAVID S.
(B) REGISTRATION NUMBER: 34,235
(C) REFERENCE/DOCKET NUMBER: 44981

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 523-3400
(B) TELEFAX: (617) 523-6440

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCTAGAAGCC CCAAGCTTAC CACCTGCA 28

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTAGATCAG GGGTTGGCCA CGATGGTGTC CTTGATCCAC T 41